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Insight into the pattern of heavy-metal accumulation in lichen thalli

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ABSTRACT

Background: Heavy metals that pass through the plasmalemma are expected to influence on lichen metabolic processes; however, lichens may tolerate high concentrations of metals by sequestrating them extracellularly. Heavy metal accumulation level fundamentally determine the success of lichens in the colonisation of polluted sites; however, the proportions between extra- and intracellular metal concentrations in lichen thalli are still poorly recognized. In this study metal accumulation patterns of selected toxic trace elements, i.e. Pb, Cd, and micronutrients, i.e. Zn, Cu and Ni, in *Cladonia cariosa* thalli were recognised in relation to extra- and intracellular fractions.

Methods: The intracellular and total concentrations of Zn, Pb, Cd, Cu and Ni in lichen thalli collected from eleven variously polluted sites were determined by means of atomic absorption spectrometry. Additionally, organic carbon and total nitrogen contents as well as pH of soil substrate were measured.

Results: The accumulation patterns differed between studied metal elements; the major part of Zn, Pb and Cd loads was accumulated extracellularly, whereas Cu and Ni accumulation was mostly intracellular. Like toxic trace elements, Zn was accumulated mainly extracellularly at high polluted sites. The non-linear models most reliably reflect relationships between intracellular and extracellular metal contents in *C. cariosa* thalli. The intracellular contents of Zn, Pb, Cd and Cu increased slower at higher than at lower extracellular concentrations. Moreover, at higher total concentrations of elements in the thalli, their extracellular proportions were markedly increased.

Conclusion: The results suggest that in the face of extreme Zn-enrichment, lichens demonstrate the ability to accumulate the excess of Zn outside the cells. Therefore, it can be concluded that metal accumulation depend not only on the element but also on its abundance in the environment and direct availability for lichens. The studied species showed a defence against excessive intracellular accumulation when a given element is in excess. Such capability may facilitate the colonization of extremely polluted sites by certain pioneer lichens.

1. Introduction

Lichens are a symbiotic association composed of a fungus and an alga and/or cyanobacterium [1]. Both nutrients and toxic elements can be absorbed directly through the surface of thallus [2] and thus lichens are sensitive to various pollutants occurring in their environment [3]. Their unique biology and sensitivity makes them highly responsive to spatial and temporal variations in atmospheric pollution. For this reason, they have been widely used as bioindicators of anthropogenic changes (e.g [4,5].) and for monitoring heavy metal pollution levels in the environment [6]. Nevertheless, many lichen species are well adapted to various unfavourable habitat conditions [7] and occur both in inhospitable natural environments and anthropogenic sites [8–11]. Despite the potential toxicity of heavy metals, some lichens appear insensitive to soils extremely rich in heavy metals [12,13].

Heavy metal accumulation level fundamentally determine the success of lichens in the colonisation of polluted sites. Lichens take up elements through three basic mechanisms: physical trapping of particulates in the intercellular spaces of the medulla, extracellular and intracellular accumulation [14,15]. Heavy metals that pass through the plasmalemma are expected to influence on lichen metabolic processes [16–18]. However, lichens may tolerate high concentrations of metals by sequestrating them extracellularly [19]. Cations of metals can bind to extracellular sites of the mycobiont and photobiont cell walls and it is one of the most important mechanism preventing the input of toxicants into cells [20]. Moreover, binding metals with organic acids, oxalate crystals, lichen secondary metabolites, polysaccharides and melanin pigments is another relatively well known detoxification mechanism [21–23]. Elements immobilized in cell wall constituents or otherwise extracellularly located are considered less toxic to lichens. The proportion of extra- and intracellular metal concentrations in lichen thalli depend on the species and element [24,25]; however, the differences in

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the share of these two forms of accumulation are still poorly recognized.

Intracellular uptake depends on the permeability properties of the cell membrane and the amount of extracellular ligands [2]. Cations with a very high affinity for extracellular ligands are almost exclusively extracellularly encountered in lichen thalli, whereas those with a low affinity to ligands are mainly found intracellularly [4]. Most heavy metal elements have intermediate attributes, but there is a lack of more detailed knowledge in this respect. The situation is more complicated since a ionic competition between Cd^{2+}, Cu^{2+}, Pb^{2+} and Zn^{2+} for binding sites of lichen thalli of *Xanthoria parietina* has been demonstrated [24]. Consequently, simultaneous exposure to these elements resulted in a decreased uptake of each metal compared with the uptake measured when each metal was supplied alone. Therefore, knowledge on general pattern of heavy-metal accumulation in lichens naturally growing in polluted environment seems to be helpful in understanding their metal tolerance.

This study aimed to recognize the pattern of Zn, Pb, Cd, Cu and Ni accumulation in thalli of epigeic lichen *Cladonia cariosa*. This pattern was examined in the following aspects: (1) the proportions of intra- and extracellular metal concentrations in relation to particular elements and soil substrate pollution; (2) regression models describing the relationships between intra- and extracellular metal concentrations in the thalli. The following hypothesis was set: toxic trace elements (i.e. Pb and Cd) were accumulated mainly extracellularly, whereas in the case of micronutrients (i.e. Zn, Cu and Ni) the intracellular fraction dominated.

2. Materials and methods

2.1. Target lichen species

Cladonia cariosa (Ach.) Spreng. is an epigeic lichen species with characteristic club-shaped and longitudinally fissured secondary thallus, crowned with brown apothecia [26]. It exemplifies a pioneer lichen in post-industrial habitats associated with the mining and processing of Zn-Pb ores [9,27]. The selected lichen proven to be a rapid and effective coloniser of bare heavy-metal-polluted soils and often significantly contributes to the biomass at strongly affected sites [12].

2.2. Study area

The study was conducted in the Silesia-Cracow Upland, one of the most polluted regions in Poland associated with the metallurgical industry [28]. Long activity of ore processing plants resulted in great heavy-metal pollution of surrounding soils [29–33]. Altogether eleven heavy-metal polluted sites were selected. Ten of them included different types of post-industrial dumps and psammophilous grasslands located in the vicinity of smelters. The eleventh was regarded as a reference site (Site 8) and constituted semi-natural psammophilous grassland not directly influenced by Zn-Pb industry but located in the same geographical region (see Fig. 1 and Table 1). The sampling sites represent wide range of soil pollution, but are characterized by similar climatic conditions (according to the data included in: [10–12,34–36]).

2.3. Sampling and lichen identification

Sampling was done in early autumn 2018. Ten lichen samples were collected at each sampling site (see Table 1). The thalli were packed into paper bags and transported to the lab. Each sample was analysed in terms of lichen secondary metabolites composition by means of standard thin-layer chromatography (TLC), following Orange et al. [37]. Lichen samples retained for further examination were chemically homogeneous and contained atranorin and rangiformic acid as major compounds (see also [38]). Additionally, three soil samples from each study site were collected for analysis of chemical parameters.

2.4. Element concentrations in lichen samples

Podetia (secondary thallus) were included in the chemical analyses. Macroscopic foreign materials adhering to thalli were carefully removed with a soft bristle brush. One part of lichen materials was designated for measurements of total element concentrations (n = 110) and the second part for intracellular concentrations (n = 110). The samples of the first part were rinsed with deionised water to remove fine particulate matter on the lichen thalli (cf [39]). Ca. 100 mg of lichen thalli of the second part were soaked in 10 mL of a 20-mM Na_2EDTA, shaken for 1 h in a vibration shaker (Vibramax 100, Heidolph) to remove metals non-specifically bound to the cell walls (cf [22,24,40]). Then the samples were dried at 90 °C for 24 h to a constant weight. Subsequently, all powdered lichen samples were digested in 70 % $HClO_4$ (Merck, Suprapur) and 65 % HNO_3 (Merck, Suprapur) (1:4) and diluted with double-distilled water. Concentrations of Zn, Pb, Cd, Cu and Ni were determined by means of atomic absorption spectrometry, using a Varian AA280FS and a Varian AA280Z with a GTA 120. Additionally, certified reference material (SRM 1570a, Spinach Leaves, NIST) was used; all measurements fell within ± 5% of the certified value (Table S1). The extracellular concentrations were calculated as the difference between total concentrations and concentrations after EDTA washing (treated here as a fraction of metals extracellularly bound with exchange sites on the cell walls of both symbionts and trapped in the form of solid particles in intercellular spaces).

2.5. Soil chemical analysis

The soil samples were dried and passed through a 2-mm sieve. pH was measured in 1 M KCl with a HQ40d pH meter (Hach Lange, Poland). Organic carbon content was measured using the dry combustion technique with a LECO SC-144DR Analyzer (LECO Corp., MI, USA) and total N content using the Kjeldahl method with a Kjeltec 2300 Analyzer Unit (FOSS Tecator, Sweden). 5 g DW samples were digested with 70 % $HClO_4$ (Merck, Suprapur) using a digester (FOSS Tecator 2020, Sweden). Subsequently, flame atomic absorption spectrometry was applied; Varian 280 Fast Sequential Atomic Absorption Spectrometer (Varian, Australia) for Zn, Cd, Pb, and Cu and Varian Zeeman 280 Atomic Absorption Spectrometer with a 120 Graphite Tube Atomizer (Varian, Australia) for Ni. Certified reference material (CRM048 – 50 G, Sandy soil, Sigma-Aldrich, WY, USA) was used for quality assurance. The recovery of measured element concentrations was in the range of 96.5–99.0% of the certified values (Table S1).

2.6. Statistical analysis

The Kruskal-Wallis tests followed by Dunn's post hoc tests were applied to verify the differences in soil chemical parameters between study sites. Then cluster analysis based on hierarchical clustering routine, the Bray-Curtis similarity coefficient and an unweighted pair-group average (UPGMA) clustering algorithm was applied in order to group study sites according to heavy-metal concentrations in soil substrate. Based on the results of this analysis, study sites were classified into two general soil pollution classes, namely: 'high polluted' and 'low polluted'. Following Levene's test, used to assess the equality of variances, Student's t-tests were performed in order to reveal significant differences in intracellular and total heavy-metal concentrations in lichen thalli across the two distinguished soil pollution classes.

The correlations between intracellular and total concentrations of elements in lichen thalli were tested with the Pearson correlation coefficients. The proportions of metals accumulated intracellularly as well as extracellularly were calculated in relation to their total content. Separate one-way analyses of variance followed by Tukey's (HSD) tests were performed in order to reveal significant differences in proportion of element accumulated intracellularly across particular metal elements on each study site. Prior to the analysis, the normality of the

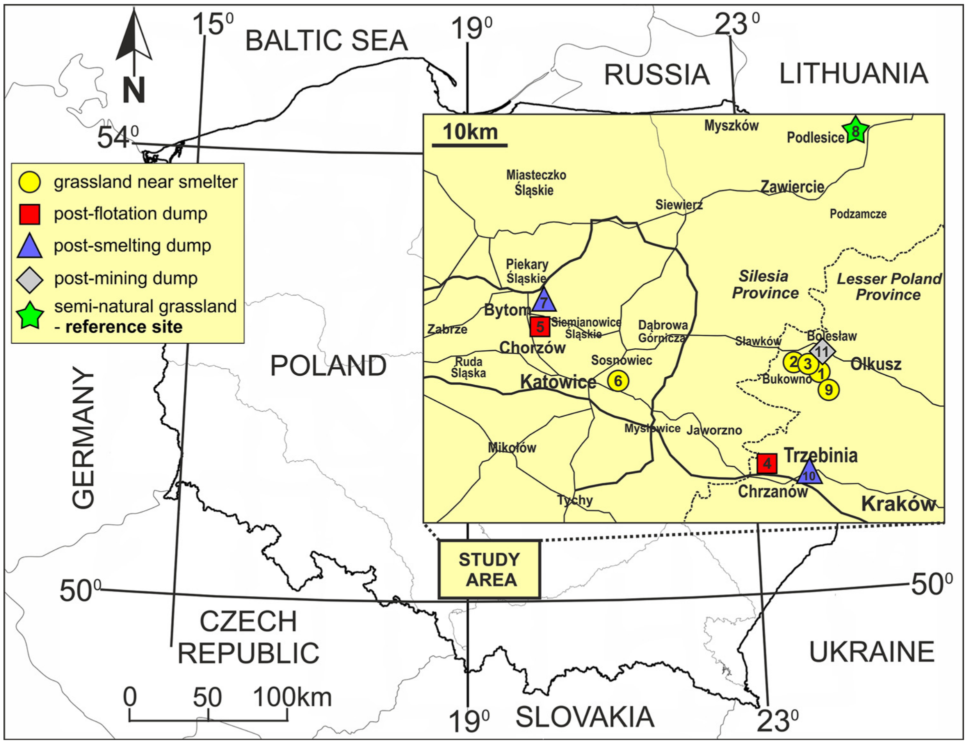

Fig. 1. Location of the study sites in the Silesia-Cracow Upland (Poland). The type of habitat for particular study sites are provided on the map. For detailed characteristics of study sites see Table 1.

distribution was verified using the Kolmogorov-Smirnov test and Levene's test was performed to assess the equality of variances. Subsequently, Student's t-tests were performed to reveal significant differences in the proportion of metal elements accumulated intracellularly in lichen thalli between the two soil pollution classes.

The relationships between intracellular and extracellular concentrations of Zn, Pb, Cd, Cu and Ni in lichen thalli were tested with various regression models (linear and nonlinear) to find the best fitted one. For this purpose, the variables were appropriately transformed to convert curvilinear models into linear models, in which parameters could be determined by least squares estimation [41]. The models that were best fitted were selected according to the coefficient of determination (R^2) (see [42]). Once the model has been fit a detailed residual analysis was performed in order to validate the regression model, obtain reliable regression coefficients and detect the outliers (extreme cases). If studentized residuals greater than 3 in absolute value (correspond to points more than 3 standard deviations from the fitted model) were detected, the outliers were excluded from the analysis.

3-D contour plots presenting three-dimensional surface on a two-dimensional plane were created to visualise the relationships between intra- and extracellular concentrations of particular elements (predictor variables) and percentage of element accumulated extracellularly (response variable shown as contours). The curves were fitted to the data according to the distance-weighted least squares smoothing procedure.

The calculations and statistical procedures were performed using STATISTICA 13 (StatSoft, Tulsa, OK), STATGRAPHICS CENTURION 18 (StatPoint, Inc) and PAST 3.22 [43].

Table 1
The sites included in the study with specification of the locality and chemical parameters of the soil substrate (mean ± SD; n = 3). The different letters next to mean ± SD values indicate statistically significant differences between study sites ($p < 0.05$) according to the Kruskal-Wallis and Dunn's post hoc tests.

Study site	Location	Zn (mg/kg)	Pb (mg/kg)	Cd (mg/kg)	Cu (mg/kg)	Ni (mg/kg)	pH	N_{tot} (%)	C_{org} (%)
Site 1	Bukowno town	1560.5 ± 279.6^{ab}	206.7 ± 13.5^{ab}	7.2 ± 0.8	15.5 ± 6.1^{ab}	5.2 ± 2.8	6.8 ± 0.2	0.08 ± 0.03	1.3 ± 0.1
Site 2	Bukowno town	460.5 ± 250.0^{ab}	196.8 ± 70.5^{ab}	5.7 ± 1.6	7.8 ± 4.4^{ab}	83.0 ± 82.8	5.9 ± 0.5	0.2 ± 0.2	3.3 ± 2.6
Site 3	Bolesław/Bukowno town	6245.2 ± 4047.0^{ab}	1776.0 ± 984.3^{ab}	62.6 ± 24.8	10.4 ± 6.2^{ab}	4.4 ± 5.2	6.7 ± 0.7	0.1 ± 0.1	1.6 ± 0.8
Site 4	Balin village	7305.9 ± 3596.2^{ab}	2253.6 ± 654.5^{ab}	64.0 ± 26.4	12.7 ± 4.5^{ab}	1.2 ± 0.3	7.6 ± 0.0	0.1 ± 0.0	1.0 ± 0.3
Site 5	Chorzów town	11899.5 ± 5728.5^{ab}	3286.1 ± 1619.3^{ab}	64.8 ± 30.6	30.4 ± 4.3^{ab}	13.6 ± 2.2	6.9 ± 0.2	0.3 ± 0.1	9.8 ± 4.0
Site 6	Katowice town	3576.5 ± 1110.1^{ab}	352.2 ± 130.5^{ab}	27.0 ± 6.7	66.9 ± 30.3^{ab}	45.1 ± 34.2	7.1 ± 0.4	0.1 ± 0.0	1.3 ± 0.5
Site 7	Piekary Śląskie town	6412.4 ± 4107.7^{ab}	5933.8 ± 4440.5^{b}	34.5 ± 18.0	129.3 ± 60.6^{b}	20.2 ± 6.7	6.3 ± 0.4	0.3 ± 0.1	6.6 ± 1.2
Site 8	Podlesice village	35.2 ± 16.1^{a}	8.3 ± 2.4^{a}	0.4 ± 0.1	1.8 ± 0.6^{a}	8.3 ± 6.9	5.8 ± 0.7	0.07 ± 0.04	0.8 ± 0.4
Site 9	Pustynia Starczynowska	199.8 ± 138.7^{a}	112.9 ± 54.3^{ab}	2.7 ± 0.6	11.8 ± 7.3^{ab}	9.2 ± 7.1	5.2 ± 1.3	0.1 ± 0.1	1.4 ± 1.2
Site 10	Trzebinia town	48035.6 ± 12539.1^{b}	5063.2 ± 1029.7^{b}	49.9 ± 23.0	216.0 ± 54.7^{b}	71.6 ± 20.3	5.9 ± 0.4	0.2 ± 0.1	3.4 ± 1.8
Site 11	Ujków Stary village	2336.7 ± 2210.4^{ab}	410.6 ± 387.3^{ab}	12.2 ± 14.0	11.1 ± 7.7^{ab}	6.1 ± 2.2	6.9 ± 0.1	0.1 ± 0.0	1.1 ± 0.8
	Mean contents for sandy soil in Poland [1]	41.7	14.2	0.29	10.4	5.5			
	Max acceptable level [2]	1000	600	15	600	300			

[1] after Kabata-Pendias and Pendias [44].
[2] Maximum acceptable levels for post-industrial areas in Poland (Regulation of the Minister of Environment [45]).

3. Results

3.1. Soil heavy metal pollution

Soil chemical parameters of the study sites are provided in Table 1. The concentrations of elements associated with processing of Zn-Pb ores, i.e. Zn, Pb and Cd, were a few to several times higher at the polluted sites compared to the reference site. The element concentrations in soil substrate of all polluted sites greatly exceeded mean concentrations recognized for sandy soils in Poland [44] and usually were far above the acceptable levels specified for post-industrial areas [45]. The reference site was relatively less contaminated, especially with Cu and Ni (Table 1), and metal concentrations were lower than the acceptable levels designated for post-industrial areas [45]. The soil pH ranged from slightly acidic to neutral at all studied sites. The soil samples, with the exception of samples from Site 5, were generally characterized by low organic carbon and total nitrogen contents.

The cluster analysis distinguished the study sites into two distinct groups (Fig. S1). Consequently, soil samples were assigned to one of two classes related to heavy-metal soil pollution, i.e. 'low polluted' (Sites 2, 8 and 9) and 'high polluted' (Sites 1, 3–7, and 10–11).

3.2. Intracellular and total metal accumulation in lichen thalli

The total and intracellular metal concentrations in the thalli of *C. cariosa* collected at particular sampling sites are provided in Table 2. The lowest concentrations of each element were determined at reference site. Regarding soil pollution classes, the results of Student's *t*-test showed that lichens collected at sites assigned to high polluted class accumulated significantly higher amounts of heavy metals (both total and intracellular) compared to lichen samples collected at sites assigned to low polluted class ($p < 0.05$). The only exception applied to intracellular Ni concentrations (Fig. S1).

The correlation matrix for intracellular and total metal concentrations in lichen thalli is presented in Table S2. The concentrations of each element were strongly and positively associated with each other. Moreover, both total and intracellular concentrations of heavy metals correlated significantly and positively with each other with the exception of relationship between intracellular concentration of Ni and intracellular/total concentration of Pb.

3.3. Heavy metal accumulation pattern

The results of one-way ANOVA revealed significant differences in the proportions of element accumulated intracellularly across particular metal elements at each study site with the exception of Site 7 (Table S3). As a rule, higher proportions of extracellular metal concentrations were found for Zn, Cd and Pb compared to Cu and Ni (Fig. 2). In case of majority of study sites, more than a half of the total load of Cu and Ni was accumulated intracellularly; whereas the proportion of Zn, Pb and Cd accumulated intracellularly did not exceed half of the total content irrespective of the study site (Fig. 2). According to the results of Tukey's (HSD) test, a significantly higher proportion of elements accumulated intracellularly was recorded for Cu and Ni in comparison to Zn, Pb and Cd at most of the study sites (Fig. 2). Regarding the data obtained from all study sites, the proportions of Zn, Pb and Cd accumulated intracellularly were significantly lower than intracellular proportions of Cu and Ni. Significantly the highest proportion of intracellular accumulation were found for Ni (Fig. 2).

Comparing heavy metal accumulation pattern at sites classified to low and high polluted classes, it turned out that proportions of Pb and Cd accumulated intracellularly were higher on average by 3% and 5% at high polluted sites then at low polluted sites (Fig. 3), however the differences were not significant (Student's t-tests; $p > 0.05$). In contrast, intracellular proportions of Zn, Cu and Ni were lower on average by 6%, 17 % and 5.5 %, respectively, at high polluted sites compared to low polluted ones and significant differences were found for Zn and Cu (Student's t-tests; $p < 0.05$).

3.4. Relationships between intracellular and extracellular metal concentrations

The visualisation and regression equations of the studied relationships are provided in Fig. 4 and Table S4. Among all tested models, nonlinear relationships in case of each studied element turned out to be the best-fitted (Table S4). The correlation coefficients and results of analysis of variance for particular selected models are provided in Table S5. Multiplicative function for Zn ($R^2 = 51.16$ %), Pb ($R^2 = 62.27$ %) and Cu ($R^2 = 33.32$ %) and S-curve model for Cd ($R^2 = 53.02$ %) describe the relationships in a most reliable way. By following the course of the curves depicting these functions, it can be generalized that along with increasing extracellular content of Zn, Pb, Cd and Cu in the lichen thalli, the increase rate of intracellular concentrations gradually decreases approximately in accordance with the provided functions (Fig. 4). Simultaneously, the proportion of elements accumulated extracellularly increases, which is most apparent at highest total metal concentrations (Fig. 4). The correlation coefficients indicated moderately strong relationships between intracellular and extracellular concentrations of Zn, Pb, Cd and Cu and weak relationships for Ni. The results of ANOVA indicated that in case of Ni there was no statistically significant relationship (Table S5).

Table 2
Descriptive statistics (mean ± SD; n = 10) for intracellular (INT) and total (TOT) metal concentrations (μg/g) in the lichen thalli of *Cladonia cariosa*. For detailed characteristics of study sites, see Table 1 and Fig. 1.

Element	Zn (μg/g)		Pb (μg/g)		Cd (μg/g)		Cu (μg/g)		Ni (μg/g)	
Study site	INT	TOT	INT	TOT	INT	TOT	INT	TOT	INT	TOT
Site 1	632.5 ± 249.8	1586.4 ± 488.3	107.7 ± 46.8	304.2 ± 111.9	5.4 ± 2.0	13.7 ± 5.7	15.0 ± 4.2	25.0 ± 6.7	4.5 ± 1.0	6.1 ± 1.4
Site 2	382.0 ± 131.1	1027.2 ± 91.9	35.0 ± 29.7	147.9 ± 25.7	1.8 ± 0.8	6.5 ± 0.9	10.3 ± 2.9	14.6 ± 1.4	4.0 ± 1.7	3.7 ± 0.5
Site 3	960.4 ± 432.3	2367.0 ± 336.5	99.3 ± 50.4	297.2 ± 62.5	5.2 ± 2.4	12.7 ± 2.4	17.7 ± 4.5	30.5 ± 5.2	6.1 ± 0.9	7.6 ± 1.4
Site 4	193.2 ± 96.7	495.1 ± 140.6	75.9 ± 63.3	231.0 ± 95.2	2.1 ± 1.4	4.7 ± 1.6	6.8 ± 1.7	11.8 ± 3.9	1.2 ± 0.2	1.1 ± 0.1
Site 5	791.2 ± 399.8	2725.9 ± 1180.1	478.8 ± 228.4	1252.2 ± 482.6	6.7 ± 4.7	20.8 ± 9.7	8.4 ± 1.1	14.8 ± 3.5	1.9 ± 0.6	4.3 ± 1.3
Site 6	142.6 ± 29.7	601.2 ± 129.6	18.7 ± 4.2	75.1 ± 12.8	1.5 ± 0.5	5.4 ± 1.1	14.0 ± 1.3	25.8 ± 2.2	1.1 ± 0.3	2.1 ± 0.2
Site 7	304.0 ± 145.1	811.0 ± 399.7	563.8 ± 210.4	1655.0 ± 470.4	2.5 ± 1.3	8.1 ± 3.1	21.6 ± 4.7	43.1 ± 9.9	2.9 ± 0.8	5.5 ± 2.4
Site 8 (reference site)	53.3 ± 14.7	114.5 ± 20.9	10.1 ± 5.9	30.4 ± 8.1	0.2 ± 0.2	0.8 ± 0.2	4.8 ± 1.5	6.8 ± 2.4	0.3 ± 0.2	0.6 ± 0.2
Site 9	123.1 ± 44.2	301.1 ± 41.9	21.5 ± 10.1	71.4 ± 9.3	0.9 ± 0.5	2.3 ± 0.9	6.1 ± 1.0	8.0 ± 0.7	2.9 ± 4.4	1.7 ± 0.2
Site 10	376.4 ± 201.2	1849.1 ± 678.4	61.0 ± 81.9	476.4 ± 321.5	0.8 ± 0.4	6.3 ± 2.1	9.8 ± 4.6	27.5 ± 4.8	1.0 ± 0.5	4.3 ± 1.1
Site 11	366.1 ± 107.4	934.8 ± 147.1	148.6 ± 56.0	379.6 ± 81.8	3.3 ± 0.9	8.2 ± 1.8	9.2 ± 3.7	15.8 ± 4.0	2.5 ± 0.7	3.2 ± 0.6

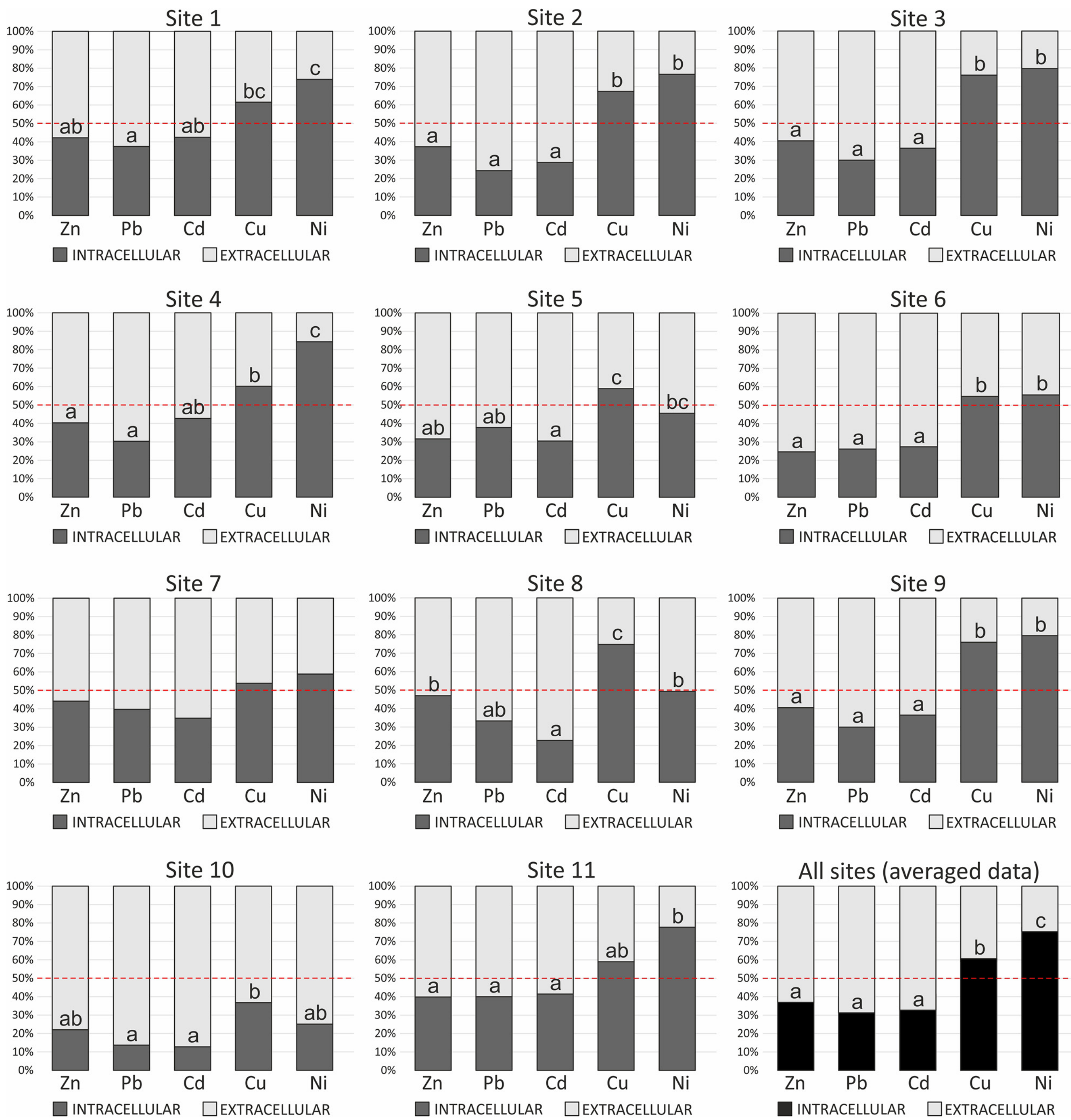

Fig. 2. Mean proportions (n = 10) of intracellular and extracellular metal element concentrations in lichen samples from particular study sites as well as all study sites including the results of one-way ANOVA for differences in the proportions of metals accumulated intracellularly. The different letters above the bars indicate significant differences ($p < 0.05$) according to post-hoc Tukey's test; see Table S3 for detailed results of ANOVA.

4. Discussion

Many factors influence the uptake of metals by lichens, and include habitat conditions, such as temperature, soil pH, soil aeration, fertilization, the availability of the elements in the soil [46]. Comparing the accumulation capacity of *C. cariosa* with other lichen species examined within the same geographical area it turned out that, at the most polluted sites, *C. cariosa* accumulated relatively higher metal concentrations in the thalli than various epiphytic lichen species (compare Table 2 with [47,48]). Although *Cladonia* lichens have been recognized as weak accumulators of heavy metals [35], high concentrations of elements reported in the present study indicate that strong soil pollution significantly contributes to increased accumulation and constitutes the main source of elements in epigeic lichens (cf [36,49]).

The accumulation patterns in *C. cariosa* thalli differed between studied metal elements. The major part of Zn, Pb and Cd loads was located outside the cells and, as a rule, more than half of their total content was associated with extracellular accumulation. This may explain why this species tolerates the excess of metals in the environment. Extracellular deposition of elements is considered as a defence mechanism that reduces intracellular accumulation of toxic substances [19]. On the other hand, Cu and Ni accumulation was mostly intracellular and well over half of the total load of these elements was located inside the cells (Fig. 2). The studied metals can be classified into two groups: (1) elements essential for lichen metabolism (Zn, Cu, Ni) and (2) toxic trace elements (Pb, Cd). It is known that toxic elements, such as Pb and Cd, have a strong affinity for cell-wall ligands. In contrast, Zn, Cu and Ni are essential micronutrients which easily penetrate

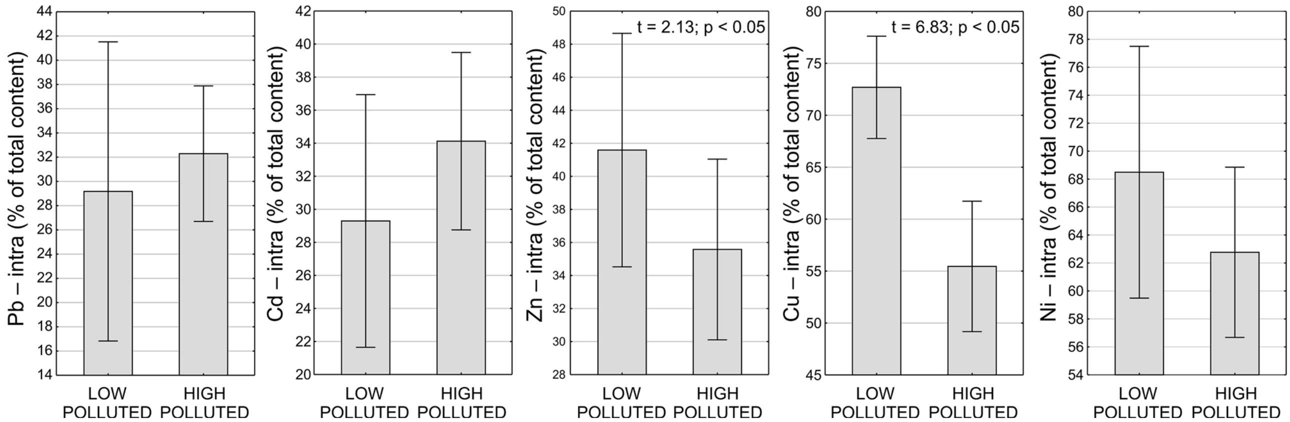

Fig. 3. The proportion of intracellular (intra) metal element concentrations (mean ± SD) in lichen samples from low and high polluted study sites including the results of Student's-t tests for differences between two soil pollution classes; t and p values are shown only for significant results ($p < 0.05$).

the plasma membrane [19,50,51]. These elements are components of several enzymes [52], behaves as an activators of the enzymes [50], and inherently penetrate the plasmalemma more easily than Pb and Cd, which results in their greater accumulation inside cells. This is in accordance with the results of present study with the exception of Zn for which the tendency to extracellular accumulation has been revealed. Such result does not comply with previous studies showing that Zn is mainly stored in the intracellular fraction in lichens [53,54]. This may be due to the fact that the Zn contents, both in the soil and accumulated inside the thalli, were exceptionally high in comparison to the Cu and Ni contents, especially when the polluted sites are considered. Presumably, in the face of extreme Zn-enrichment, lichens may demonstrate the tendency to accumulate the excess of Zn outside the cells. Therefore, it can be concluded that heavy metal accumulation pattern depend not only on metal element but also on its prevalence in the environment and availability for lichens (Fig. 3). It is noteworthy that the proportion of Zn accumulated intracellularly proved to be significantly higher at low polluted sites compared to high polluted ones. This could explain the dual pattern of Zn accumulation and gives the answer why Zn is accumulated in a similar way to toxic trace elements, such as Pb and Cd, at high polluted sites.

Two processes can be responsible for high extracellular Zn concentration in lichen thallus. The first process can be related to complexation of heavy metals. For example, Pb and Zn in *Diploschistes muscorum* are accumulated through an enhanced synthesis of oxalate, which precipitates toxic elements as insoluble salts outside the cells [21,55]. In the latter case, solid Zn-containing particles originating from post-industrial wastes could be simply and efficiently trapped in the intercellular spaces of medulla which contributes to the increase in the proportion of extracellular fraction. This is likely since secondary thallus of *C. cariosa* is densely branched, fissured longitudinally along the podetia length and covered by discontinuous cortical layer [26]. Such kind of growth form facilitates the interception and retention of particles inside the thallus [56]. The amount of metal accumulated by the lichen is species dependent and refers to its morphological and structural features [57]. Most of study sites are located near zinc smelters which emits large amounts of Zn-containing particles and post-industrial dumps generate a lot of Zn-rich dust. Consequently, continual entrapment of Zn-rich particles by fungal hyphae during lichen growth could constantly occur (see also [58]). This may also support the explanation of the increased extracellular accumulation of Zn in *C. cariosa* thalli.

Differences between the two revealed patterns in relation to the dominance of intracellular or extracellular fraction in lichen thalli can also be explained by the fact that the Cu and Ni contents in soil substrate samples, and consequently in the lichen thalli, were relatively low compared to the contents of Zn, Pb and Cd. The study sites can be considered, at most, as slightly contaminated with these elements compared to another Cu-Ni polluted areas (see e.g. [59,60]). Therefore, even though Cu and Ni are elements that easily pass through the cell membrane, it cannot be ruled out that the pattern of their accumulation in lichen thalli would be similar to this observed for Zn, Pb and Cd at sites heavily contaminated with these metals. However, it should be mentioned here that in *Cladonia* individuals collected from Cu-rich substrata in central and eastern Slovakia, the proportion of intracellular Cu fraction clearly exceeded half of the total content [60]. Compared to *C. cariosa* examined under this study, *C. arbuscula* accumulated 2.1–7.0 times higher and *C. furcata* 3.0–11.1 times higher intracellular contents of Cu (while total contents for both species were 1.7–7.8 and 2.7–15.1 times higher, respectively) [cf 60]. This suggests an advantage of intracellular Cu accumulation in lichen thalli even at high concentrations of the metal in the surrounding environment. Nevertheless, the treatment of thalli with high Cu concentration solutions in laboratory conditions caused that the proportion of extracellular fractions became greater than intracellular [60–62]. It is also worth pointing out that extracellular Cu binding capacity can vary significantly between lichen species [61]. However, the general rule can be outlined that in less Cu-polluted sites the metal is accumulated almost entirely inside cells, whereas when high concentrations of Cu are present in the environment, the greater part of metal load is accumulated extracellularly, although it does not exceed 50 % of the total content (Fig. 3; cf [60]). Finally, the competition between metals for binding sites could be another factor affecting element accumulation in the lichen thalli [24] and the revealed proportions between extracellular and intracellular concentrations of particular elements. Paoli et al. [24] demonstrated under controlled laboratory conditions that due to this competition there is a possible risk of underestimating the real level of heavy metal pollution in the environment by using lichens in biomonitoring studies. Although the importance of this process could not have been assessed under present study due to the fact that uptake of trace elements are strongly influenced by habitat factors such as soil pH, microclimatic conditions and other external factors [19], one should keep in mind that competitive behaviour of heavy metals could be of great importance when lichens inhabiting their natural environment are exposed to many toxic trace elements simultaneously.

Although intracellular accumulation was generally correlated with extracellular, the non-linear models best reflect relationships between intra- and extracellular metal contents in *C. cariosa* thalli (Fig. 4, Table S4). The intracellular contents of Zn, Pb, Cd and Cu increased slower at higher than at lower extracellular concentrations. Moreover, at higher concentrations of elements accumulated in the thalli, the extracellular proportion of elements markedly increased. Such phenomenon was found both in case of Zn, Pb and Cd for which the proportion of extracellular concentrations clearly exceeded intracellular, as well as in

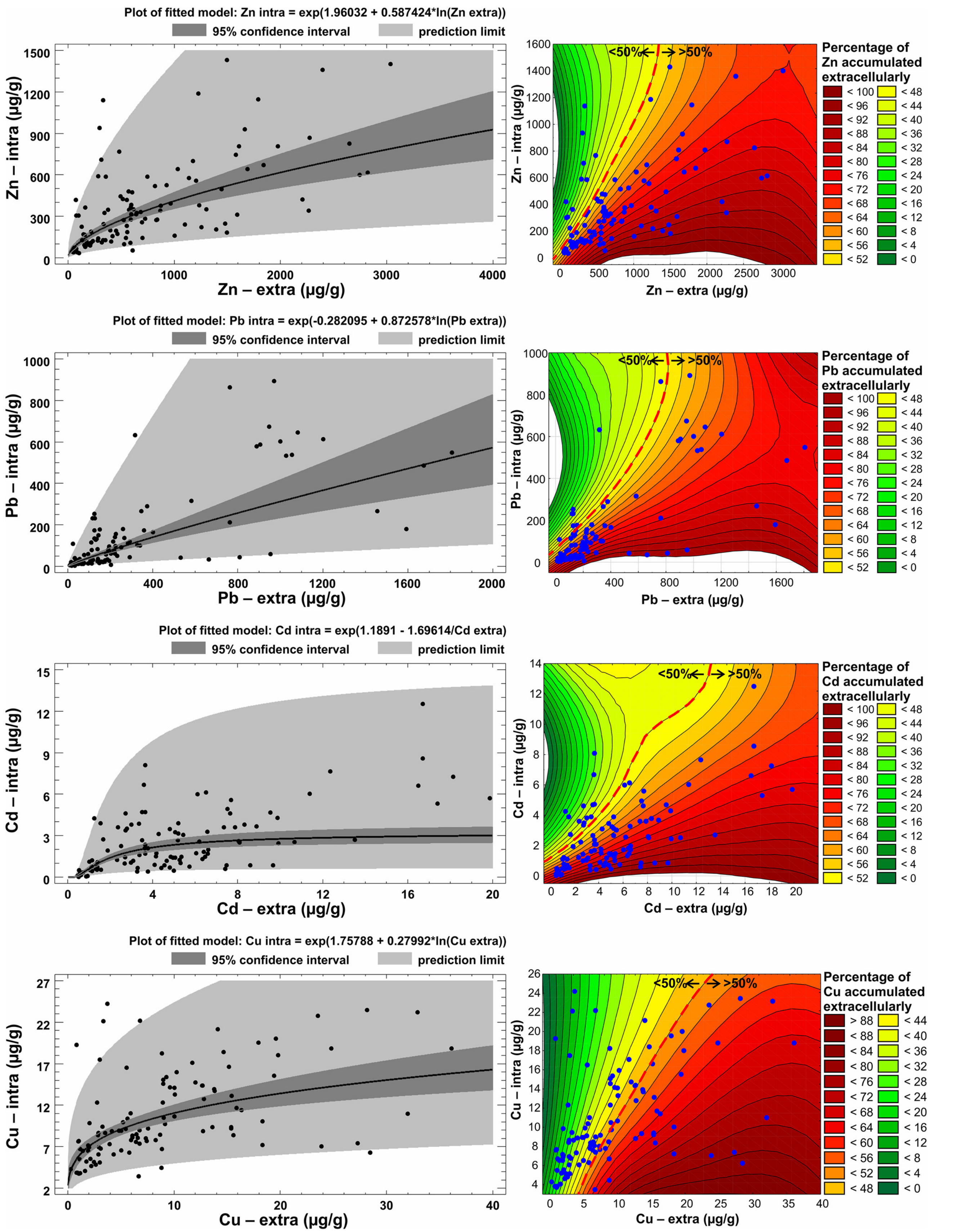

Fig. 4. Regression plots with fitted functions presenting the relationships between intracellular and extracellular Zn, Pb, Cd and Cu concentrations in lichen thalli (left side). The contour plots presenting relationships between intracellular and extracellular Zn, Pb, Cd and Cu concentrations and proportions (%) of element accumulated extracellularly (shown as contours) in lichen samples. The curves are fitted to the data according to the distance-weighted least squares smoothing procedure (right side).

case of Cu for which even if the extracellular fraction rarely exceeded 50 % of the total metal load, its proportion clearly increased at high concentrations of this metal accumulated in the thalli (Fig. 4). This suggests lichen defence against excessive intracellular accumulation when a given element is in excess. Additionally, the competitive effects of different ions may also affect intracellular uptake. Because Zn, Pb and Cd frequently co-occur in contaminated sites, competitive effects may result in low internal concentration of a single metal element [63].

5. Conclusions

This paper shows details on accumulation in lichen thalli of different heavy metal elements on the example of model species *C. cariosa*. The studied metal elements differ considerably in terms of the proportion of intra- and extracellular fraction accumulated in the lichen thalli. Moreover, the way of element deposition in the thalli depends also on the degree of soil substrate pollution. The results suggest that in the face of extreme element enrichment, lichens demonstrate the tendency to accumulate the excess of elements extracellularly, which prevent the input of toxicants into cells. Such capability may facilitate the colonisation of extremely polluted sites by certain pioneer lichens.

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CRediT authorship contribution statement

Kaja Rola: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Resources, Visualization, Writing - original draft, Writing - review & editing.

Declaration of Competing Interest

The author declares no conflict of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jtemb.2020.126512.

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